Genetic polymorphisms of paraoxonase-1 are associated with chronic kidney disease in Japanese women

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Paraoxonase-1 (PON1) is an HDL cholesterol–associated antioxidant enzyme, and some of its polymorphisms are linked with systemic oxidative stress and cardiovascular events. In this study, we genotyped seven single nucleotide polymorphisms (SNPs) within the *PON1* gene and determined their association with chronic kidney disease in 2,968 individuals from the general Japanese population. We found that a missense SNP (rs662) with a G-to-A substitution leading to an amino acid substitution (G[Arg]/A[Gln]), was significantly associated with albuminuria and estimated glomerular filtration rate (eGFR), especially in women. The A/A genotype in women had the highest prevalence of albuminuria and the lowest values of adjusted eGFR. In contrast, such relationships were not detected in men. Multivariate regression analysis found that the A/A genotype was an independent and significant factor for albuminuria and renal insufficiency (eGFR less than 60 ml/min/1.73 m^2). The serum PON1 activity was lowest in subjects with the A/A genotype. In biopsy specimens, immunohistochemical analysis found increased PON1 expression on the endothelial surface of sclerotic renal arterioles and glomerular capillaries in patients with hypertension or diabetes. Our study shows that this PON1 G-to-A substitution may be a key player in a common pathway to chronic kidney and cardiovascular diseases in women.

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KEYWORDS: albuminuria; general population; genotype; oxidative stress; paraoxonase-1; renal function

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Chronic kidney disease (CKD) is recently attracting the attention of clinicians because of high prevalence in the general population. Especially, its relation with cardiovascular diseases (CVD) observed in several epidemiological studies has a wide influence on daily practice.[1,2] The common diseases including CVD and CKD are multifactorial disorders affected by various environmental and genetic factors. The risk factor analyses for the development of renal insufficiency showed that the environmental risk factors such as hypertension, hyperglycemia, dyslipidemia, smoking, and oxidative stress are involved.[3,4] Conversely, little is known about genetic factors.

Oxidative stress is induced by various metabolic disorders and plays an important role in the pathogenesis of atherosclerosis. An antioxidative agent is considered to attenuate the progression of vascular injury. Paraoxonase-1 (PON1), one of the enzymes with antioxidative properties, is a high-density lipoprotein–associated enzyme that promotes the function of high-density lipoprotein (that is, antioxidation, anticoagulation, and anti-inflammation). It is reported that serum PON1 activity was decreased in patients with atherosclerosis, Alzheimer's disease, and chronic renal failure.[5–8] Recently, Bhattacharyya *et al.*[9] reported that the genotype of PON1 is associated with serum PON1 activity, systemic oxidative levels and cardiovascular events. These observations suggest a possibility that PON1 might be involved in a wide range of diseases induced by oxidative stress.

However, it is unknown whether the PON1 genotype is related to the development of chronic kidney disease. In this study, we examined the relation between renal function, urine albumin excretion, and PON1 in the Japanese general population.

RESULTS

Characteristics of subjects

Baseline characteristics of 2968 subjects who entered into a final analysis were as follows; mean age was 63.0 ± 10.5 years

old, 1343 men (45.2%), 1629 subjects (54.9%) with hypertension, 234 subjects (7.8%) with diabetes, 895 subjects (30.2%) with obesity, 988 subjects (33.3%) with hypercholesterolemia, and 653 subjects (22.0%) with albuminuria.

Polymorphisms in PON1 genes examined in this study

PON1 gene is located on chromosome 7q21–q22. We have selected seven single nucleotide polymorphisms (SNPs) regarding PON1 genes that displayed frequent minor allele frequencies in Japanese from dbSNP database of the NCBI and International HapMap Project as described in Materials and Methods section. The chromosomal locations of these seven SNPs are shown in Figure 1 and their characters are summarized in Table 1.

To examine linkage disequilibrium (LD) of the PON1 gene, we performed LD analysis using seven SNP typing data. We observed a consistency of genotypes between SNP5 and SNP7, rs622G/A, rs2269829C/T and rs854555T/G, by detecting a linkage disequilibrium ($D' > 0.85$ and $r^2 > 0.70$) (Figure 2). It is well known that the missense SNP5 rs662 with amino acid substitution is related to paraoxonase activity and the LD analysis demonstrated that the surrounding SNP6 and SNP7 were in LD block with SNP5 in this population. Therefore, we hereafter focused this SNP5 rs662 and examined its relation with albuminuria and renal function. The comparison of basal characters in subjects with rs662 genotypes A/A, A/G, and G/G was shown in Table 2. There was no significant difference in the basal characters among subjects with these genotypes except uric acid levels.

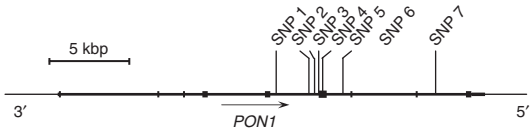

Figure 1 | The chromosomal locations of SNPs in *PON1* gene. Coding exons are represented by thick blocks connected by bold lines representing introns. The 5′ and 3′ untranslated regions are displayed as thinner blocks on the leading and trailing ends of the aligning regions. SNP1, rs3917527; SNP2, rs2057681; SNP3, rs3917538; SNP4, rs3917541; SNP5, rs662; SNP6, 2269829; SNP7, rs854555.

Association of PON1 SNP rs662 genotype with albuminuria and eGFR

First we examined the relationship between rs662 genotypes and urine albumin excretion. The prevalence of albuminuria was significantly related to rs662 genotypes with highest value in A/A genotype (A/A: 27.9, A/G: 22.2, and G/G: 21.1%, respectively, $P = 0.0327$) in total subjects. Further analysis revealed the gender difference in the relation. In women A/A genotype showed the strikingly high prevalence as compared with other genotypes (A/A: 32.5, A/G: 21.8 and G/G: 19.5%, $P = 0.0017$). In contrast, there was no significant difference between genotypes in men (Figure 3a). The levels of urine albumin–creatinine ratio showed a similar tendency that the significant relation was observed in women, but not in men (Figure 3b).

Next, we examined the relationship between genotype of rs662 and renal function. To exclude the effects of covariates we adjusted estimated glomerular filtration rate (eGFR) for age, gender, body mass index, systolic blood pressure, total protein, total cholesterol, triglyceride, uric acid, HbA1c, hemoglobin, smoking, and drinking, using a general linear model. Then, we performed quantitative trait locus analysis of population having this SNP and adjusted eGFR by an analysis of variance (ANOVA). This analysis showed that adjusted eGFR levels were significantly associated with rs662 genotypes in women. The eGFR values of population having SNP rs662 A/A, A/G, and G/G were 78.7 ± 0.5, 79.5 ± 0.2, and 80.2 ± 0.2 ml/min per 1.73 m^2 (mean ± s.e.), respectively ($P = 0.0139$). Again, such a relation was not observed in men (Figure 4). These relationships between renal function, albuminuria, and rs662 genotype were preserved in non-diabetic population after excluding diabetic subjects (data not shown).

Further, to examine the independent relation of rs662 genotype with albuminuria and renal insufficiency (eGFR < 60 ml/min per 1.73 m^2), we performed multivariate logistic regression analysis including age, gender, hypertension, diabetes, obesity, smoking, drinking, and hypercholesterolemia.

Table 1 | Polymorphisms in PON1 genes examined in the study

SNP ID	NCBI SNP reference	SNP type	Public location position (B36.2)	Allele		Allele frequency		Genotype			Heterozygosity
				1	2	Allele 1	Allele 2	11	12	22	
SNP1	rs3917527	intron 5	94778194	A	G	0.88	0.12	2274	649	44	0.22
SNP2	rs2057681	intron 5	94776193	C	T	0.66	0.34	1290	1350	326	0.45
SNP3	rs3917538	intron 5	94775829	T	C	0.53	0.47	822	1492	653	0.50
SNP4	rs3917541	intron 5	94775560	C	T	0.88	0.12	2264	647	44	0.22
SNP5	rs662	missense	94775382	G(Arg)	A(Gln)	0.66	0.34	1287	1351	327	0.45
SNP6	rs2269829	intron 6	94774065	C	T	0.65	0.35	1249	1369	345	0.45
SNP7	rs854555	intron 8	94778327	T	G	0.65	0.35	1222	1335	360	0.46

NCBI, National Center for Biotechnology Information; PON1, paraoxonase-1.
allele 1, major allele; allele 2, minor allele.
Typing call rates were over 99% The Hardy-Weinberg equilibrium *P*-values did not deviate in all SNPs ($P > 0.1$).

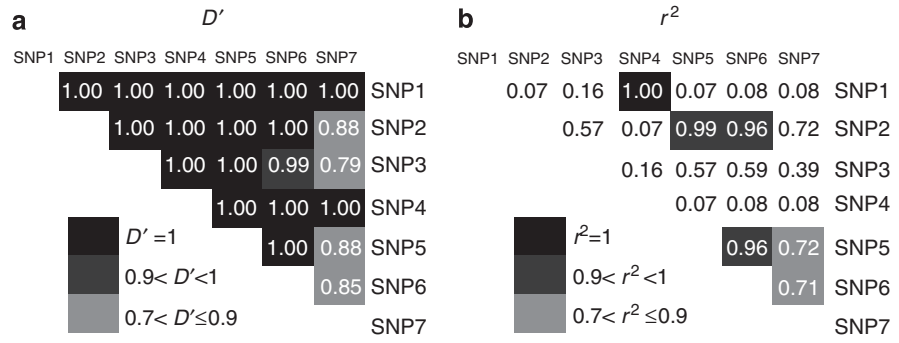

Figure 2 | Linkage disequilibrium (LD) analysis of seven SNPs in *PON1*, as measured by D' (a) and r^2 (b). Dark squares signify high LD values. SNP1, rs3917527; SNP2, rs2057681; SNP3, rs3917538; SNP4, rs3917541; SNP5, rs662; SNP6, 2269829; SNP7, rs854555.

Table 2 | Genotype-based comparisons of basal characteristics in rs662

rs662 Genotypes	A/A	A/G	G/G	*P*-value
Number	326	1350	1287	
Men (%)	50.0	44.0	45.3	NS[a]
Age (years)	62.9 ± 9.9	63.0 ± 10.3	63.0 ± 10.3	NS[b]
Drinker (%)	45.4	40.0	41.9	NS[a]
Current smoker (%)	20.2	17.4	19.1	NS[a]
Hypertension (%)	55.2	53.9	55.8	NS[a]
Diabetes (%)	7.7	7.7	8.2	NS[a]
Hypercholesterolemia (%)	32.5	33.8	32.9	NS[a]
Obesity (%)	30.7	29.6	30.5	NS[a]
Systolic BP (mm Hg)	134.7 ± 15.1	133.6 ± 16.1	134.7 ± 15.7	NS[b]
Diastolic BP (mm Hg)	79.9 ± 9.7	78.9 ± 10.2	79.6 ± 9.9	NS[b]
Serum creatinine (mg/100 ml)	0.70 ± 0.16	0.67 ± 0.16	0.68 ± 0.29	NS[b]
Hemoglobin A1c (%)	5.25 ± 0.70	5.24 ± 0.65	5.25 ± 0.69	NS[b]
HDL-C (mg/100 ml)	59.4 ± 14.9	59.2 ± 14.8	58.9 ± 14.1	NS[b]
LDL-C (mg/100 ml)	123.5 ± 29.0	125.0 ± 30.3	123.5 ± 29.3	NS[b]
Triglyceride (mg/100 ml)	108.0 ± 77.0	106.4 ± 63.5	106.4 ± 60.1	NS[b]
Uric acid (mg/100 ml)	5.2 ± 1.4	5.1 ± 1.3	5.0 ± 1.4	0.015[b]
Hemoglobin (g/100 ml)	13.8 ± 1.6	13.7 ± 1.4	13.7 ± 1.4	NS[b]
Past history of CVD (%)	12.3	11.1	13.7	NS[a]

BP, blood pressure; CVD, cardiovascular disease; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol; NS, not significant.
[a]χ^2 test.
[b]Analysis of variance, Mean ± s.d.

It demonstrated that the rs662 A/A genotype was an independent factor for albuminuria (odds ratio 1.432 (95%CI 1.091–1.879), $P = 0.0096$) and renal insufficiency (odds ratio 1.503 (95%CI 1.016–2.224), $P = 0.0412$) (Table 3).

The paraoxonase activity in rs662 genotype

To confirm the relationship between rs662 genotypes and the enzymatic activity of PON1, we measured paraoxonase activities in 150 subjects that their basal characters were matched (A/A: $n = 60$, A/G: $n = 45$, and G/G: $n = 45$, respectively). It revealed that paraoxonase activity was related to rs662 genotype and the A/A genotype had the lowest activity (Figure 5). This relation was observed both in men and women. We also examined the levels of malondialdehyde-LDL, one component of oxidized LDL in these subjects. However, there was no significant difference among them (A/A: 145.6 ± 50.6, A/G: 160.0 ± 50.6, and G/G: 165.3 ± 50.2 U/L [mean ± s.d.], $P = 0.1170$).

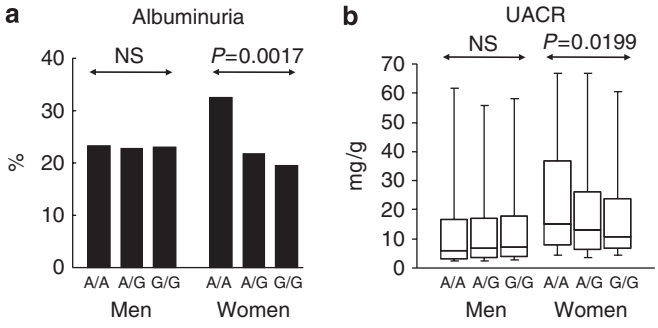

Figure 3 | The association between rs662 genotype and urine albumin excretion. (**a**) The prevalence of albuminuria. *P*-value shows the comparison among rs662 genotypes by a χ^2 test. (**b**) Urine albumin–creatinine ratio. *P*-value shows the comparison among rs662 genotypes by non-parametric Kruskal–Wallis test. NS; not significant.

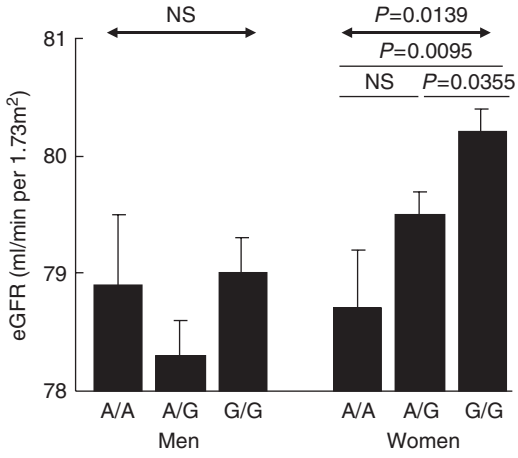

Figure 4 | Genotype-based association of PON1 with eGFR. Estimated GFR was expressed as mean ± s.e. *P*-value shows the comparison among rs662 genotype by analysis of variance with Bonferroni test as a *post hoc* test. NS; not significant.

The localization of PON1 expression in human renal specimen

Finally, we investigated the localization of PON1 expression in human kidney using immunohistochemical method. In human biopsy specimen, the expression of PON1 was strongly detected in vascular endothelial surface in sclerotic

Table 3 | Odds ratio of rs662 genotype for albuminuria and renal insufficiency (eGFR <60 ml/min per 1.73 m^2)

	Unadjusted		Adjusted[a]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Albuminuria				
rs662 A/A	1.397 (1.076–1.813)	0.0119	1.432 (1.091–1.879)	0.0096
Renal insufficiency (eGFR <60 ml/min per 1.73 m^2)				
rs662 A/A	1.427 (0.977–2.083)	0.0657	1.503 (1.016–2.224)	0.0412

OR, odds ratio.
[a]Adjusted for gender, age, drinking, smoking, obesity, diabetes, hypertension, and hypercholesterolemia.

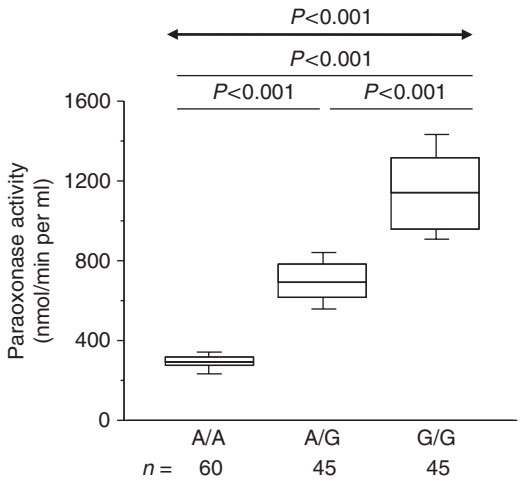

Figure 5 | Comparison of the levels of paraoxonase activity between rs662 genotypes. *P*-value shows the comparison among rs662 genotype by ANOVA with the Bonferroni test as a *post hoc* test.

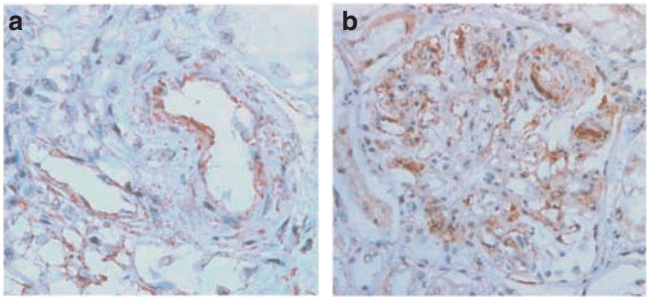

Figure 6 | Immunohistochemistry for PON1 in human biopsy specimens. (**a**) 54-year-old male with benign nephrosclerosis, magnification $\times$ 400, (**b**) 74-year-old female with diabetic nephropathy, magnification $\times$ 200.

renal arterioles and glomerular capillaries in subjects with hypertension (Figure 6a) and diabetes (Figure 6b). In normal specimen obtained from renal tumor section, PON1 expression was scarcely observed (data not shown).

DISCUSSION

In this study, we have revealed for the first time the association between albuminuria, renal function, and PON1 genotype in the Japanese female population. This result suggests that genetic variation of PON1 might affect the development of chronic kidney disease as well as cardiovascular diseases.

The evidence that PON1 has an antioxidant effect and vasoprotective properties, has been provided by studies using transgenic mice.[5,6] Furthermore, the relation of serum PON1 activity with vascular damages was reported in several clinical studies.[10,11] Chronic kidney diseases belong to a category of vascular diseases, therefore, it is speculated that PON1 might play a part in the development of CKD. This study showed that the PON1 genotype was associated with estimated GFR and the prevalence of albuminuria in women. A histological study revealed a PON1 expression in damaged renal vessels. These findings suggest that PON1 genotype might affect vascular damage by modulating its antioxidative capacity and could be one of the common genetic factors for various kinds of vascular diseases.

The PON1 activity is mostly regulated by its genotypes and the SNP rs662 genotype accompanying amino acid substitution is related to its enzymatic activity (high in G/G genotype and low in A/A genotype).[12] Theoretically, SNP rs662 A/A genotype has a lower antioxidative effect and less vasoprotective property. However, which genotype of PON1 rs662 is a risk factor for cardiovascular diseases has been controversial,[13] because of a lack of the study that concomitantly examined PON1 genotype, its enzymatic activities, blood oxidative status, and vascular damages in humans. Recently, Bhattacharyya *et al.*[9] revealed a systematic evidence that PON1 genotype SNP rs662 A/A was related to lower PON1 activity, higher plasma oxidative stress, and poor CVD prognosis. In accordance with Bhattacharyya's report, our result showed that rs662 A/A genotype with a low paraoxonase activity was a risk factor for albuminuria and renal insufficiency in women. These findings suggest that rs662 A/A genotype might be used as a common risk allele for CKD and CVD.

In the development of CKD, the interaction between environmental and genetic factors plays an important role. A previous study has revealed the significance of environmental factors including hypertension, diabetes, obesity, and smoking.[3] In contrast, the analysis in a genetic effect is still limited. Pinto-Sietsma *et al.*[14] has reported the association between Endothelin-1 genotype and renal impairment in general non-diabetic population in Netherlands. In our previous study, we found that the genotype of inflammatory chemokine, CC chemokine ligand 5 is associated with urine albumin excretion.[15] This study further showed that the PON1 genotype was independently related to albuminuria and renal function even after adjusting for conventional risk factors. These observations suggest that the genetic factor might be involved in the development of CKD in a general population by affecting renal vascular damages. Of note, there is a possibility that PON1 genotype might have an indirect pathway to induce vascular damages by modulating other risk factors such as lipid metabolism and blood

pressure levels. The adjustment with these risk factors might underestimate the effect of PON1 on the development of CKD.

As reported earlier *in vivo* and *in vitro*,[12,16,17] the close relation between PON1 genotype and its activity was confirmed in this study. Therefore, it is speculated that PON1 genotype is linked to renal injury through the levels of PON1 activity. At first, we hypothesized that PON1 might affect vascular damage by modulating the oxidation of lipids. However, the relation of PON1 genotype with Malondialdehyde–low-density lipoprotein levels was not detected suggesting that Malondialdehyde–low-density lipoprotein might not be the main target of its antioxidant activity. The PON1 has a potential to hydrolyze many substrates including a variety of oxidized lipids,[9] therefore other substrates or mechanism might be involved in its vasoprotective effect.

The reason why the relations between PON1 genotype and CKD were detected only in women is unknown. However, a similar finding was documented by Christiansen *et al.*[18] that rs662 genotype was related to a survival rate only in women, but not in men. The risk factors for renal injury such as hypertension, diabetes, and smoking were more common in men than in women and might abrogate the beneficial effect of PON1. Especially, smoking is reported to reduce PON1 activity.[19] In this population the prevalence of current smokers were higher in men (34.3%) than in women (5.4%). These factors or other gender-associated factors might alter the relation of PON1 with the susceptibility to CKD. To clarify this point further study is necessary.

There are several limitations in this study. First, the subjects with serious diseases including stroke and myocardial infarction are usually followed by their doctors and do not attend this community-based health check. Therefore, the effect of PON1 genotype on CVD might be underestimated in this study. This might be one of the possible reasons that the relationship between SNP rs662 genotype and CVD described in previous studies[20–22] was not observed in this population. Second, this finding was obtained from our single cohort. To confirm the effect of PON1 on CKD it should be verified in another independent cohort. However, the allele frequency of rs662 greatly depends on ethnicity and the frequency of A allele is much lower in Japanese (0.34) than other ethnicities (0.69–0.76).[23] Therefore, the relation between PON1 genotype and CKD might be modified by ethnicities.

The precise mechanisms how PON1 attenuate the vascular damage is not fully understood; however, the overexpression of PON1 significantly reduces atherogenic lesions in the animal model.[6] This suggests that PON1 might be a potential therapeutic target for the prevention and treatment of CKD as well as CVD in future.

In conclusion, this study revealed that PON1 genotype was related to albuminuria and renal function, possibly through the modulation of renal vascular damage. The PON1 might be one of the key players in the common pathway to CKD and CVD.

MATERIALS AND METHODS

Study population

This study is part of the comprehensive 'Molecular epidemiological study utilizing the regional characteristics' of 21st Century Centers of Excellence (COE) and global COE Program in Japan as previously described in detail.[24] The aim of this study is to determine the association between genetic variants and CKD in a general population in Japan. This study is a design-incorporated baseline survey that consisted of a self-administered questionnaire on lifestyle, blood pressure measurement, anthropometrical measurement, and collections of blood and urine specimens from participants at annual health checkup. Genomic DNA was extracted from peripheral blood samples.[15]

The survey population in this study is the general population over 40-year-olds in Takahata, Japan. In 2004 and 2005, a total of 3115 subjects (mean age 63; men 1380; women 1735) took part in the program and agreed to join the study. This study was approved by the institutional ethical committee. All participants gave written informed consent.

Among 3115 subjects, 146 subjects were excluded from the present analysis due to their incomplete data. Thus, 2968 subjects were entered into final analyses (mean age 63 years; men 1343; women 1625).

Measurement

Clinical information concerning medical history, current medication, smoking habits, and alcohol intake was obtained from a self-reported questionnaire. Blood pressures were determined by using a mercury manometer, in subjects who had rested in a sitting position for at least 5 min before the measurement. Hypertension was defined as systolic blood pressure $\geqslant$140 mm Hg and/or diastolic blood pressure $\geqslant$90 mm Hg, and/or the use of antihypertensive medication. Body mass index was calculated from weight and height measures as weight (kg) divided by the square of height (m^2). Obesity was specified as body mass index $\geqslant$25.0 kg/m^2 both in men and in women. Diabetes was ascertained either by self-reported physical diagnosis or by a measure of fasting blood sugar $\geqslant$126 mg/100 ml or HbA1c value $\geqslant$6.5%. The subjects with impaired glucose tolerance were included in this analysis. Hypercholesterolemia was ascertained by a measure of serum total cholesterol $\geqslant$220 mg/100 ml, and/or the use of antihyperlipidemic medication. Urine albumin–creatinine ratio was calculated from a single spot urine specimen collected in the morning. Urine albumin concentration was determined by an immunoturbidimetry. Albuminuria was defined as urine albumin–creatinine ratio $\geqslant$20 mg/g in men and $\geqslant$30 mg/g in women, respectively.[25] Serum creatinine was measured by an enzymatic method and eGFR was obtained using the modified MDRD equation with Japanese modification.[26] The paraoxonase activity was measured by the rate of generation of *p*-nitrophenol from paraoxon (BML Inc., Tokyo, Japan).[27] Malondialdehyde—low-density lipoprotein was examined by ELISA (SRL Inc., Tokyo, Japan).

SNP selection and genotyping

We have utilized dbSNP database of the NCBI (http://www.ncbi.nlm.nih.gov/SNP/) and International HapMap Project (http://www.hapmap.org/index.html) to extract all SNPs of PON1 gene with a minor allele frequency greater than 0.1 in the Japanese general population. Seven SNPs were selected and have been genotyped. All SNPs gave accurate typing (call rate >99%) and were used in this study.

Genotypes for these seven SNPs were determined by an Invader assay (Third Wave Technologies, Madison, WI, USA)[28,29] and TaqMan allelic discrimination assay.[30] Reagents were purchased from Applied Biosystems (Foster City, CA, USA). TaqMan probes were designed and synthesized by Applied Biosystems, and distinguish the SNPs at the end of a polymerase chain reaction. One allelic probe was labeled with fluorescent FAM dye and the other with the fluorescent VIC dye. Polymerase chain reaction was performed by TaqMan Universal Master Mix without UNG (Applied Biosystems) with polymerase chain reaction primers at a concentration of 900 nM and TaqMan MGB probes at a concentration of 200 nM. Reactions were performed in 384-well formats in a total reaction volume of 3 μl using 3.0 ng of genomic DNA. The plates were then placed in a GeneAmp PCR System 9700 (Applied Biosystems) and heated at 95°C for 10 min, followed by 40 cycles at 92°C for 15 s and 60°C for 1 min, with a final soak at 25°C. The plates were read by the Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity in each well of the plate was read.[28] Fluorescence data files from each plate were analyzed by the SDS 2.0 allele calling software (Applied Biosystems). Several data (signal intensity) were eliminated to preserve the reliability of the assay system (missing data are guaranteed to be less than 1%).

Statistical analyses

We used a χ^2 test to evaluate differences in proportions and an ANOVA with the Bonferroni test as a *post hoc* test to evaluate differences in means. For some of the clinical and biochemical traits that did not distribute normally, we applied a non-parametric Kruskal–Wallis test. To confirm the Hardy–Weinberg equilibrium among genotypes, a χ^2 test was used ($P \geqslant 0.05$). LD for the combination of variations was tested by D' and r^2 by using Haploview. Data are expressed as mean ± s.d. except as otherwise indicated. A significant difference was defined as $P < 0.05$. All statistical analysis was performed using SPSS version 15.0.1J (SPSS Inc., Chicago, IL, USA).

Immunohistochemistry

Anti-PON1 goat antibody and peroxidase-conjugated donkey anti-goat immunoglobulin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Immunohistochemical staining was performed on frozen sections of human biopsy specimen using enzyme-labeled antibody method. Frozen sections were air-dried and fixed in acetone for 5 min. Endogenous peroxide activity was quenched by incubating sections in 0.3% H_2O_2/methanol for 20 min. Sections were incubated with an antibody against PON1 (dilution 1:100) at 4°C overnight. After incubating with secondary antibody at a concentration of 1:100 for 1 h, immunoreaction products were developed using 3,3′-diaminobenzidine as the chromogen, with standardized development times. Sections were then counterstained with hematoxylin acetate. Negative controls were prepared by using irrelevant normal goat IgG as a primary antibody.

DISCLOSURES

All the authors declared no competing interests.

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